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EXHIBIT A

Differentiation of Vascular Endothelium in the Chick Chorioallantois: A Structural and Autoradiographic Study[1]

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The maturation of vascular endothelial cells in the chick chorioallantoic membrane, from 8 to 18 days after fertilization, was investigated by light and electron microscopy. Light microscopic autoradiography following administration of tritiated thymidine was used to determine the thymidine labeling index of the endothelial cell population at various stages of development. Results indicate that, prior to day 11 of incubation, endothelial cells have the morphological characteristics of immature and relatively undifferentiated cells. During this time they exhibit a high labeling index of approximately 23%. At 11 days, the labeling index decreases to 2.8%, and subsequently the cells begin to acquire structural characteristics of mature differentiated endothelium. The pattern of endothelial cell labeling suggests that, during the period of high endothelial cell mitosis, the capillary network of the growing chorioallantoic membrane is expanding by an overall proliferation of endothelial cells in existing capillaries, rather than by formation of new capillary sprouts. The immaturity of endothelial cells in the young chorioallantois, or conversely their high rate of cell division, may influence the ability of this membrane to support grafted tissue prior to day 11.

INTRODUCTION

The chick chorioallantoic membrane (CAM) (Hamilton, 1952; Romanoff, 1960) has been used extensively as a host for transplantation of a variety of normal and neoplastic tissues. The ability of the CAM to support the growth of such tissues apparently increases as the membrane ages, reaching an optimum from 9 to 12 days (Danchakoff, 1918; Alfthan, 1956) and has been related to the proximity of the capillary network to the explant in older membranes (Danchakoff, 1918). Studies in this laboratory have been concerned with the response of the CAM and its blood vessels to tumor implants. Since neovascularization of tumor tissue could well be influenced by the stage of development of the CAM, an examination of the structure of its vessels at various stages of incubation with particular emphasis on the fine structure of the endothelium seemed warranted.

Although the blood vessels of the developing CAM have been well described in light microscopic studies (Fülleborn, 1895; Danchakoff, 1917), until recently the fine structure of these vessels has received only brief attention. An ultrastructural description of developing CAM vessels has been provided by Sethi and Brookes (1971) and although significant structural differences were seen between chorioallantoic vessels and their immature precursors in the allantois, no further differentiation was observed after day 6 of incubation. Our studies show, however, that progressive structural changes occur in endothelial cells in the developing CAM, and these may be correlated with changes in the thymidine labeling index of the endothelial cell population.

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METHODS AND MATERIALS

Light and electron microscopy of chorioallantoic membranes. Chorioallantoic membranes were obtained from Leghorn chicken eggs 8, 10, 12, 14, 16, and 18 days after fertilization and subsequent incubation at 37°C and 60% humidity. A portion of the shell over the airspace was removed and the exposed CAM with overlying shell membrane was flooded with Karnovsky's fixative (Karnovsky, 1965), consisting of 2% formaldehyde (freshly prepared from paraformaldehyde) and 2.5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer (pH 7.4). Additional fixative was injected below the membrane into the allantoic cavity. After 10 min of fixation *in situ*, a portion of the CAM was removed and placed in fresh fixative at room temperature for an additional 2 hr. The tissue was then washed at least overnight in cacodylate buffer containing 6% sucrose, diced into 1-mm square pieces, and postfixed in similarly buffered 1% osmium tetroxide for 1.5 hr at 4°C. Tissue pieces were block stained in 0.1% aqueous uranyl acetate for 0.5 hr at room temperature, dehydrated in ethanol, and embedded in Epon 812 or Araldite.

For light microscopy 0.5-μm sections were cut with glass knives and stained with a solution of azure II and methylene blue (Graziadei and Metcalf, 1971). For electron microscopy pale gold to gray sections were cut with a diamond knife, stained with uranyl acetate followed by lead citrate (Reynolds, 1963), and examined in a Siemens Elmiskop 1A electron microscope operated at 80 kV.

Labeling of endothelial cells and autoradiography. The chorioallantoic membranes of eggs 8, 10, 12, 14, and 16 days old were exposed, a 1-mm square portion of the shell membrane was removed, and 50 μCi of tritiated thymidine (New England Nuclear Corp.; specific activity 2.0 Ci/mmole) dissolved in 0.5 ml of Medium 199 was applied over the underlying CAM. The hole in the shell was covered with transparent tape, and the egg was returned to the 37°C incubator for 5 hr. The CAM was then dissected from the egg, washed in 3 changes of Medium 199, fixed and embedded as described above.

Sections for autoradiography, 0.8 μm thick, were mounted on glass slides, dipped into Eastman-Kodak NTB-2 emulsion, and exposed for 1 or 2 weeks at −20°C. After development and fixation, slides were stained in a solution of azure II and methylene blue (Graziadei and Metcalf, 1971). The emulsion was destained by immersing the slides in 0.05% sodium bisulfite (Gahan, 1972).

At each incubation time, two eggs were prepared and two samples of tissue from each egg were sectioned for autoradiography. Autoradiographs from each sample were analyzed by counting the number of labeled and unlabeled endothelial cells in a population of approximately 250 cells. On each day studied, the labeling indices were averaged and the standard deviation from the mean was calculated. Approximately 1000 cells were counted for each of six incubation times.

RESULTS

Light Microscopy of Chorioallantoic Blood Vessels

From the earliest stage examined in this study, 8 days of incubation, the ectodermal, mesenchymal and endodermal layers of the CAM were already well formed. Blood vessels were confined to the mesenchymal and ectodermal layers and consisted of small arterioles and venules located in the mesenchyme, which in turn supplied an extensive capillary network located under the chorionic ectoderm (Romanoff, 1960). Occasional communications between the vessels of the two layers were observed. At 8 days, chorionic vessels were located immediately below the chorionic ectodermal cells (Fig. 1a) (Danchakoff, 1917).

The endothelium of the chorioallantoic

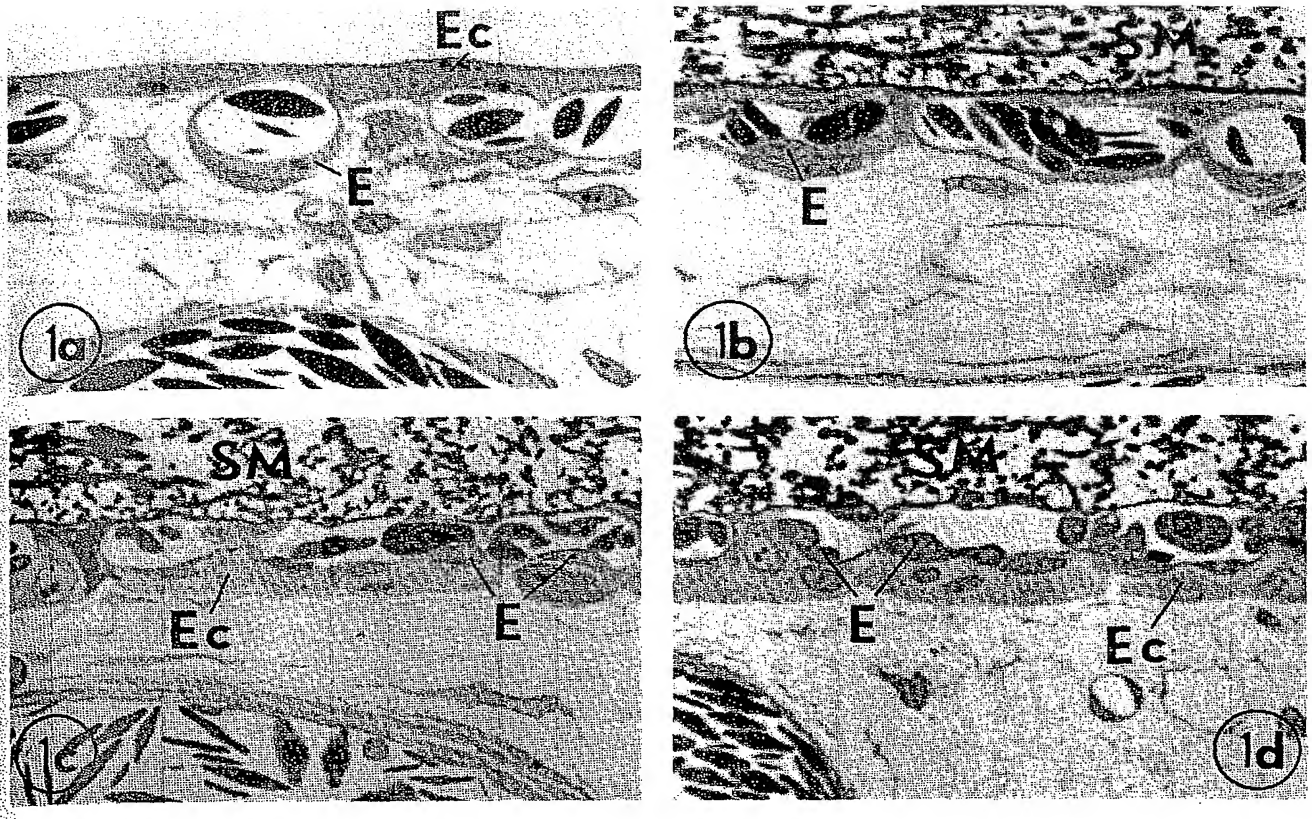

FIG. 1. Thick Epon sections of the capillary network of the chick chorioallantoic membrane at various stages of incubation. As the membrane ages, the capillary network migrates through the chorionic ectodermal layer of cells (Ec) to a position adjacent to the shell membrane (SM). (a) At 8 days of incubation, capillaries are lined with flattened endothelial cells (E) which thicken only slightly at the nucleus. The shell membrane which overlies the CAM is missing. (b) By 12 days incubation, the nuclear regions of endothelial cells (E) protrude slightly into the capillary lumen. (c) In the 14-day CAM, endothelial cell (E) nuclei protrude into the vessel lumen and are most frequently located on the side of the vessel furthest from the shell membrane. (d) At 18 days of incubation, the nuclei of endothelial cells are rounder and stain more darkly than in younger membranes. × 860.

blood vessels, especially that of the chorionic capillaries was the focus of this study. At 8 days endothelial cells were very flat, thickening only slightly at the nucleus and tapering toward their edges. In cross section, the walls of chorionic capillaries were formed by only 1 or 2 such cells. Endothelial cell nuclei were flat and elongated and protruded only slightly or not at all into the vessel lumen, so that the lumen profile was quite smooth. The nucleoplasm was lightly stained and contained a prominent nucleolus and a small amount of peripherally situated chromatin.

After 10 days of incubation, the sheet of chorionic capillaries was still located below the ectodermal cells. Endothelial cell morphology appeared unchanged from that observed at 8 days.

By 12 days of incubation, chorionic capillaries were situated closer to the shell membrane (Fig. 1b). At this stage, the first changes in endothelial cell morphology were noted. Nuclei were rounder, causing the nuclear regions of endothelial cells to protrude slightly into the vessel lumen. Increased nuclear chromatin was evident.

At 14 days, migration of chorionic blood vessels was complete so that they were now adjacent to the shell membrane (Fig. 1c) (Danchakoff, 1917). The most prominent change in endothelial cell morphology was a decrease in their thickness on the side of the vessel nearest the shell membrane.

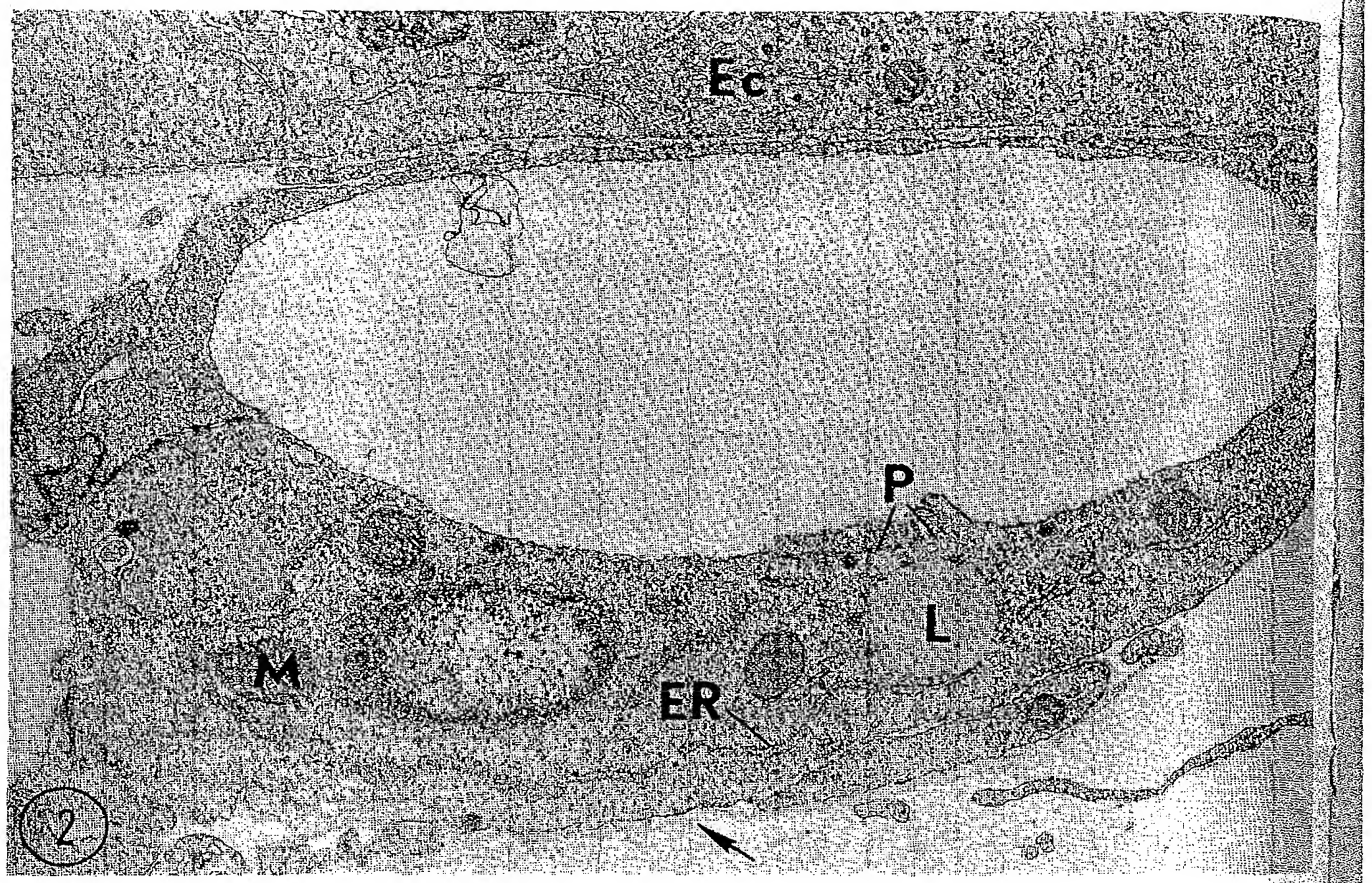

FIG. 2. Electron micrograph of a capillary from a 10-day CAM. The vessel is located below cells of the chorionic ectoderm (Ec). The endothelial cell cytoplasm exhibits many free ribosomes, rough endoplasmic reticulum (ER), scattered mitochondria (M), lipid globules (L), and a few pinocytotic vesicles (P). A subendothelial basement membrane is present in some areas (arrow). × 19,000.

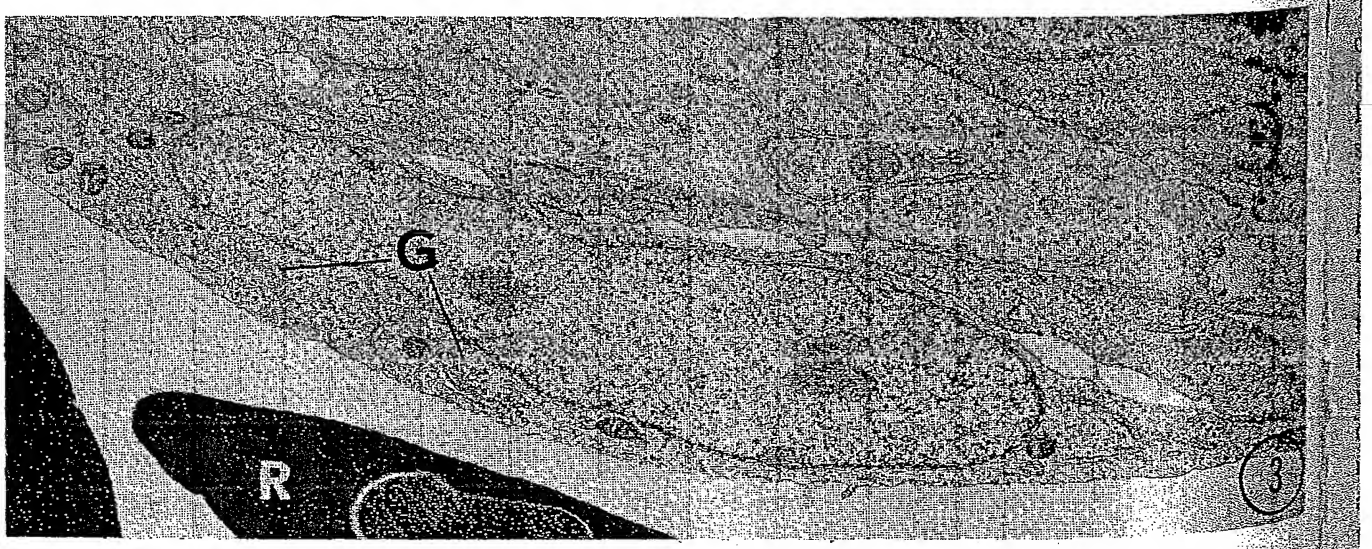

FIG. 3. Flattened endothelial cell from a 10-day CAM blood vessel. The elliptical nucleus contains a sparse amount of chromatin and is enclosed in a smoothly contoured nuclear membrane. Adjacent to the nucleus is an extensive Golgi apparatus (G). In most areas no subendothelial basement membrane is apparent. The vessel lumen contains nucleated red blood cells (R). × 11,500.

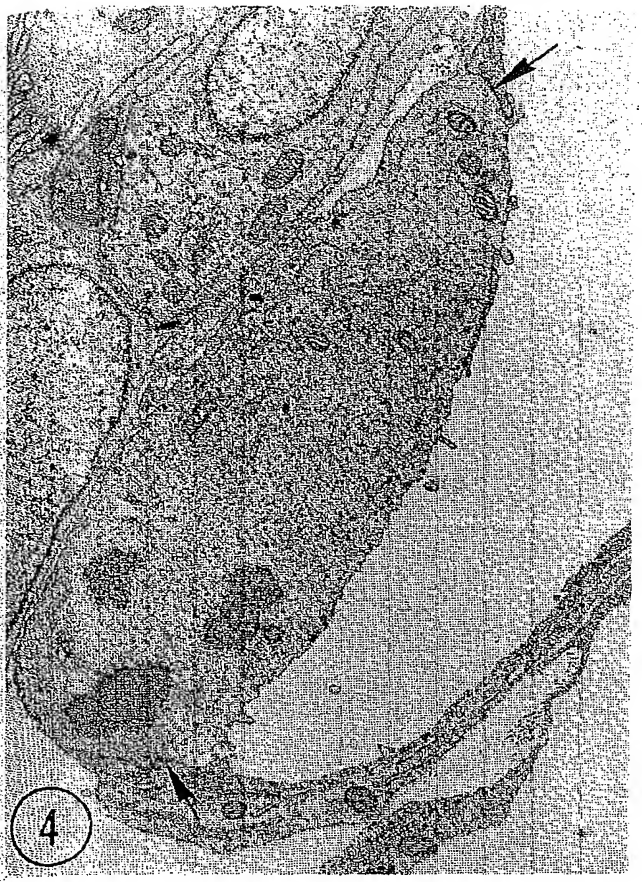

FIG. 4. Capillary of a 10-day CAM containing an endothelial cell in mitosis. Junctions between this cell and neighboring endothelial cells appear to be intact (arrows). × 9000.

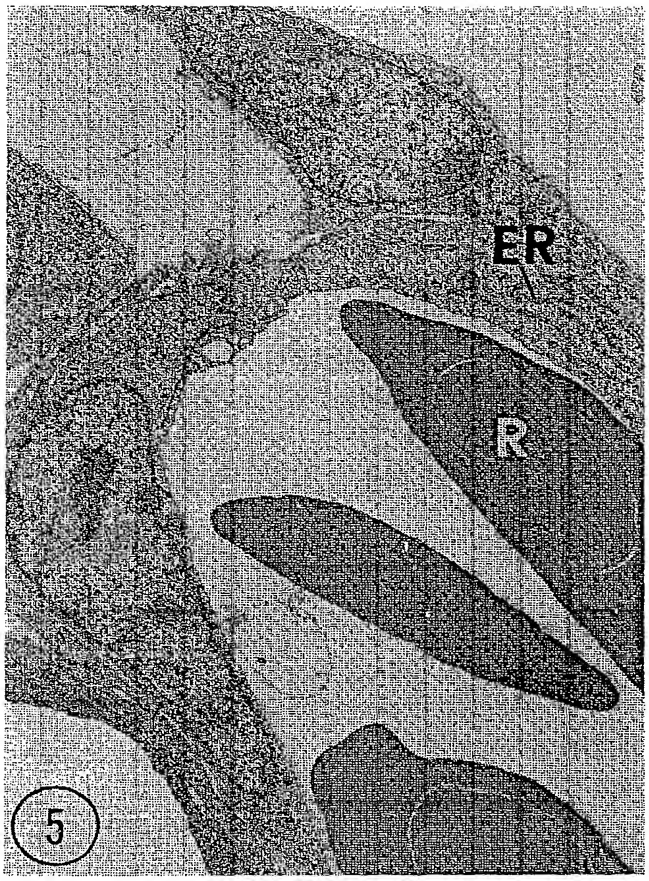

FIG. 5. Capillary from a 12-day CAM containing nucleated red blood cells (R). The endothelial cells exhibit an abundance of free ribosomes and rough endoplasmic reticulum (ER). The amount of nuclear chromatin appears to be increased when compared to endothelial cells in younger membranes. Few, if any, pinocytotic vesicles are apparent. × 7500.

This region of the endothelium usually consisted of only attenuated cytoplasmic processes. The nuclei of these cells were frequently located on the mesenchymal side of the vessel.

From 16 to 18 days of incubation, the position of the capillaries within the chorionic ectoderm remained unchanged from that observed at 14 days (Fig. 1d). Endothelial cell nuclei were noticeably rounder than those in younger membranes and the nuclear regions of endothelial cells frequently bulged into the vessel lumen. The nucleoplasm appeared more darkly stained and contained more chromatin. Endothelial cell nuclei were only infrequently located on the side of the vessel adjacent to the shell membrane.

Electron Microscopy of Chorioallantoic Blood Vessels

The progressive changes in the endothelial cells of the CAM from days 8 to 18 of incubation were confirmed and extended by ultrastructural examination of its blood vessels.

The flattened endothelial cells of the 8- and 10-day-old chorionic vessels exhibited a finely granular cytoplasm (Figs. 2 and 3) containing many free ribosomes, a few scattered mitochondria, parallel cisterns of rough endoplasmic reticulum, and a well-developed, paranuclear Golgi apparatus (Fig. 3). The nuclear membrane was smoothly contoured, exhibited no deep

invaginations and enclosed a granular lightly staining nucleoplasm (Fig. 3). Chromatin was distributed as a very thin layer adjacent to the nuclear membrane, with only a few patches of chromatin located central to this layer. Organelles occasionally observed in endothelial cells included: microfilaments, pinocytotic vesicles, and multivesicular bodies consisting of a few small vesicles (300–400 Å in diameter) enclosed in a membrane. Adjacent endothelial cells were closely apposed and sometimes overlapped for a short distance. Apposed plasma membranes sometimes exhibited increased density (Fig. 2), however, these interendothelial junctions were not studied in detail. The adventitial surface of the capillary endothelium was covered by a very thin, discontinuous basement membrane. In the 8- and 10-day-old CAM, endothelial cells in mitosis were frequently observed (Fig. 4).

At 12 days of incubation, little change in endothelial cell ultrastructure was noted except for a small increase in the amount of condensed nuclear chromatin (Fig. 5) and a decrease in the prominence of the Golgi apparatus. More striking changes were seen on day 14. Endothelial cell nuclei were less ovoid, resulting in an increased thickness in this region of the cell; nuclei frequently protruded into the vessel lumen (Figs. 6 and 7). Chromatin was more abundant and occurred in patches throughout the nucleus. Endothelial cell cytoplasm stained more darkly than that in younger chorioallantoic membranes. Pinocytotic vesicles appeared to be more numerous while rough endoplasmic reticulum was decreased. The cytoplasm also exhibited

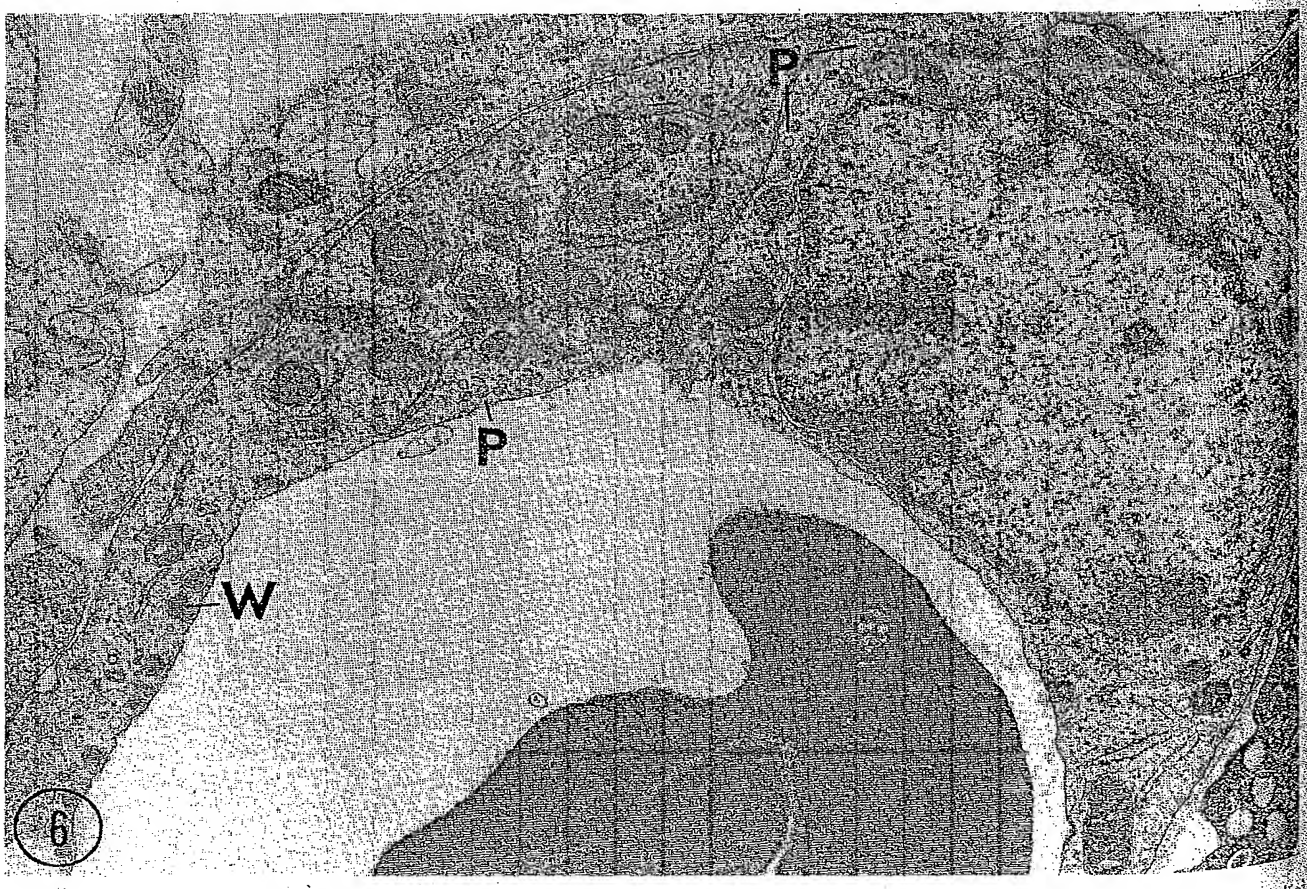

FIG. 6. Wall of a CAM capillary at 14 days incubation. In addition to the usual organelles, the endothelial cells exhibit pinocytotic vesicles (P) and Weibel-Palade bodies (W). ×20,500.

numerous organelles, first described in endothelial cells by Weibel and Palade (1964) (Fig. 6). Adjacent endothelial cells overlapped to a greater extent than in younger capillaries (Fig. 7). The basement membrane surrounding endothelial cells was still thin and discontinuous (Fig. 6). Beyond this membrane, endothelial cells were completely surrounded by chorionic ectodermal cells and their processes. Regions of close apposition were devoid of an intervening basement membrane.

Changes occurring in endothelial cells of the 16-day-old CAM were a decrease in thickness of chorionic endothelial cells nearest the shell membrane and an absence of nuclei on this side of the vessel wall. Organelles were scarce in these attenuated regions except for pinocytotic vesicles, ribosomes, and a few elongated mitochondria (Fig. 8). The nuclear membrane exhibited irregular contours.

By 18 days of incubation, endothelial cell nuclei were almost round, causing a pronounced bulging of cells into the vessel lumen (Fig. 9). The nuclear membrane exhibited deep invaginations and enclosed a more darkly staining nucleoplasm with abundant chromatin (Fig. 10). The cell cytoplasm was more darkly staining and exhibited a prominent Golgi apparatus, mitochondria, many pinocytotic vesicles

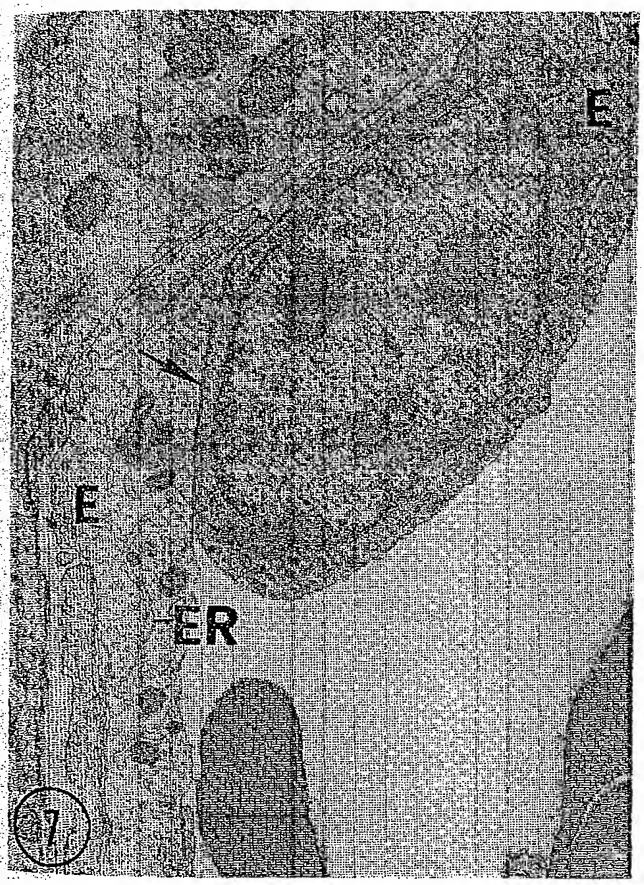

FIG. 7. Endothelial cells (E) lining an ectodermal capillary from a 14-day CAM. In its nuclear region, one endothelial cell bulges slightly into the vessel lumen. Adjacent cells are joined by a region of extensive overlap (arrow). Compared to endothelial cells in younger membranes, the nucleus is less flattened and contains more chromatin. The amount of rough endoplasmic reticulum (ER) appears to be decreased. × 10,000.

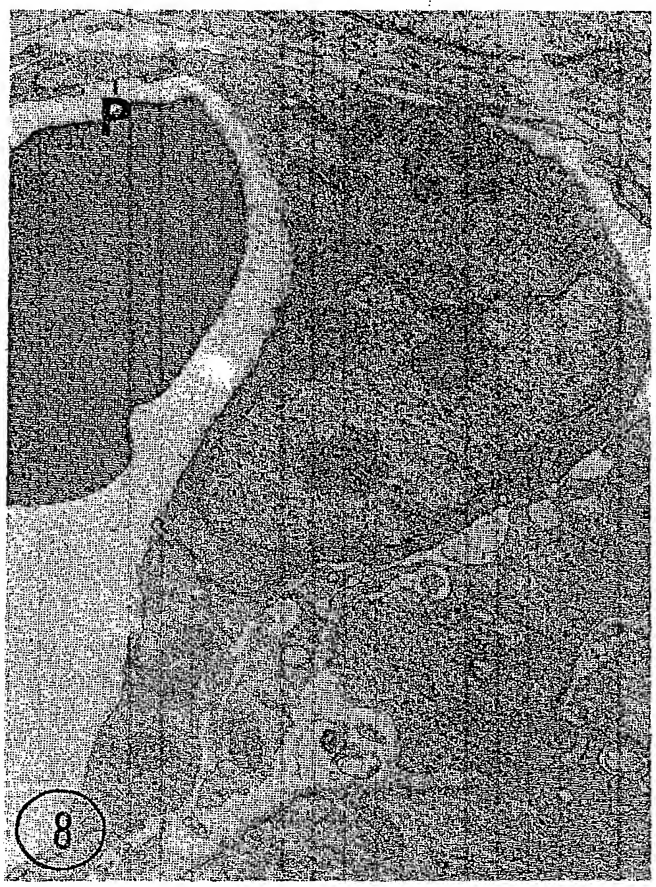

FIG. 8. Capillary endothelial cell in a 16-day CAM. The nucleus, thicker than in younger membranes, is enclosed by an irregularly contoured nuclear envelope. An attenuated region of an adjacent endothelial cell contains many pinocytotic vesicles (P). × 17,500.

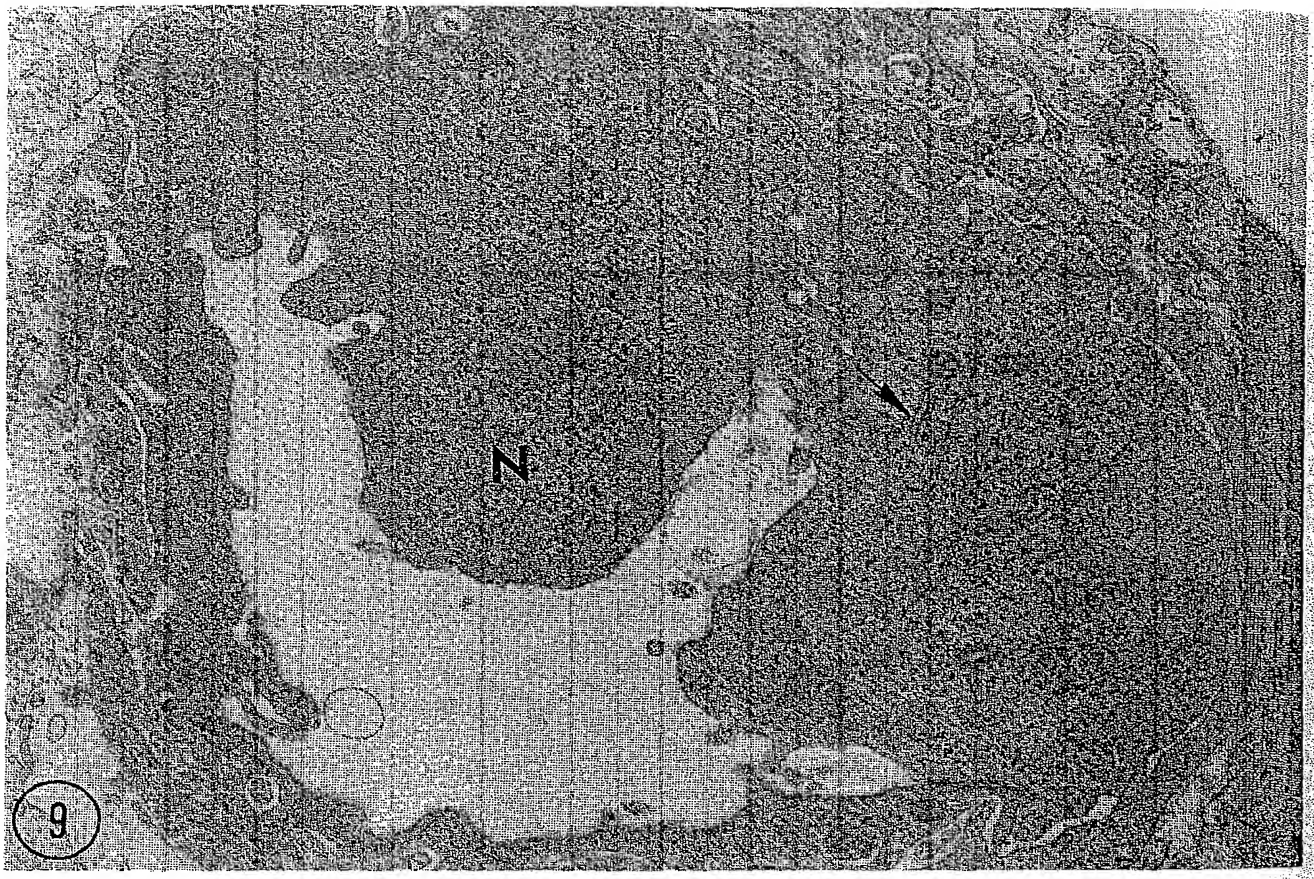

FIG. 9. Ectodermal blood vessel after 18-day incubation. In the region of the rounded nucleus (N), endothelial cells bulge prominently into the vessel lumen. Both the nuclear and plasma membranes of these cells are convoluted. One cell contains unusual paired cisternae (arrow), the surfaces of which appear devoid of ribosomes. In general, the cells of the blood vessel wall appear to stain more darkly than in younger membranes. × 13,000.

but little rough endoplasmic reticulum (Figs. 9 and 10). Endothelial cell membranes were sometimes convoluted except on the side of the cell nearest the shell membrane (Fig. 10). Cells resembling white blood cells were frequently situated in contact with the luminal surface of endothelial cells (Fig. 10).

Autoradiography of Chorioallantoic Blood Vessels

From day 8 to day 10, many cells of the CAM were intensely labeled as a result of incorporation of tritiated thymidine (Fig. 11a). Labeled cells included chorionic ectodermal cells, allantoic endodermal cells, connective tissue cells, and blood cells as well as endothelial cells. Because of the greater number of capillaries as opposed to other vessel types, most labeled endothelial cells were associated with capillaries although labeled endothelial cells also occurred in venules. In thick Epon sections, labeled endothelial cells appeared to be evenly distributed throughout the capillary network. Of approximately 1000 endothelial cells examined, 21.1% were labeled at 8 days and 23.1% at 10 days (Fig. 12).

By 11 days of incubation, the frequency of cell labeling in the CAM was greatly reduced (Fig. 11b). Only 2.8% of all endothelial cells counted and apparently incorporated tritiated thymidine (Fig. 12).

From 12 to 16 days of incubation, the proportion of labeled endothelial cells slowly increased (Figs. 11c and 12), al-

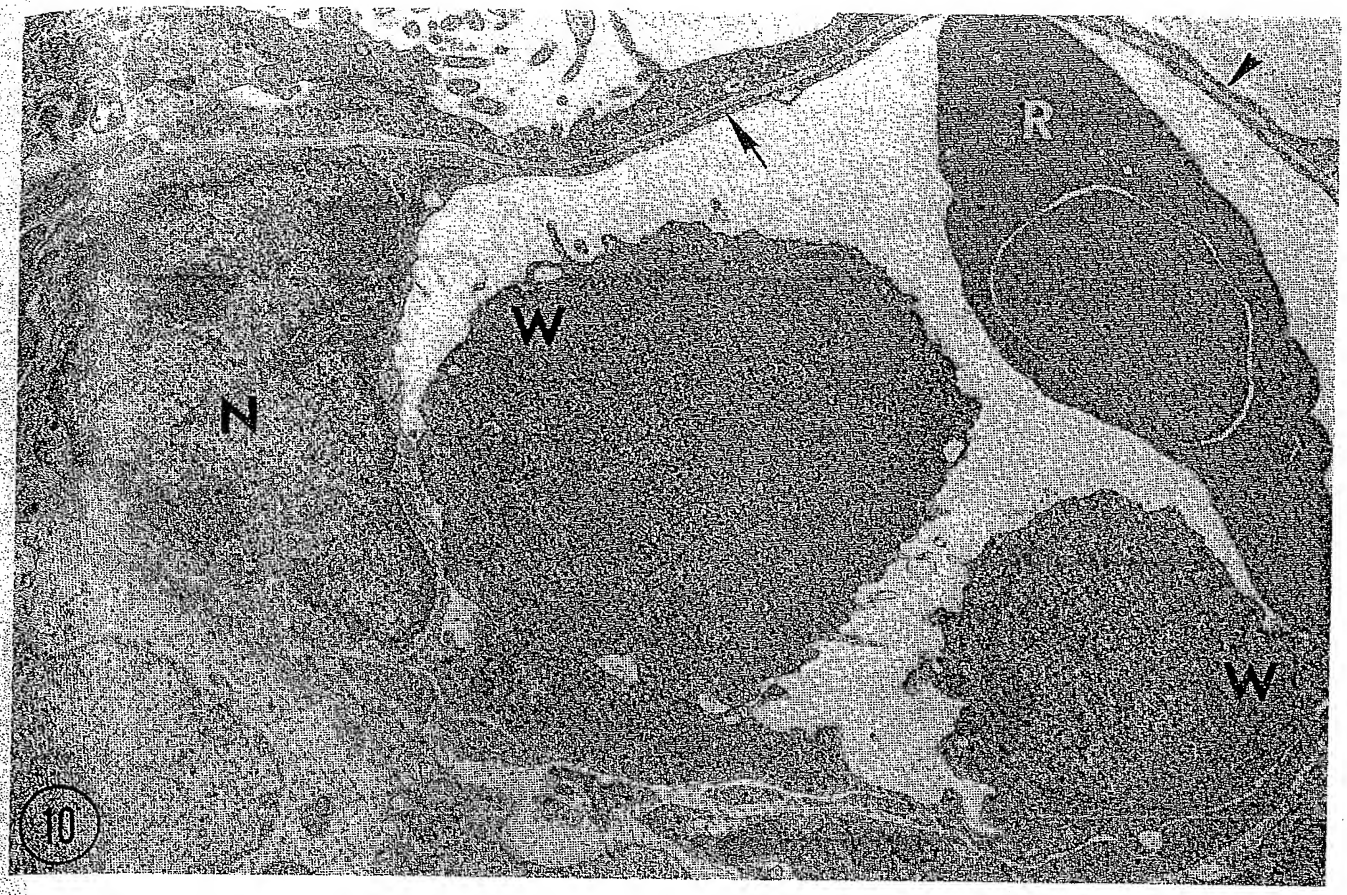

FIG. 10. Capillary in the chorionic ectodermal layer of an 18-day CAM. The endothelial cell cytoplasm is greatly attenuated (arrow) on the side of the vessel adjacent to the shell membrane. A thin ectodermal cell process (arrowhead) separates the capillary from the overlying shell membrane. The nucleus (N) of the endothelial cell contains an abundance of chromatin. Contained in the vessel lumen are a nucleated red blood cell (R) and two cells (W) resembling white blood cells, both of which are in contact with the surface of endothelial cells. × 10,000.

though never reaching the high level observed prior to day 11. Increased labeling also occurred among the other cells of the CAM.

DISCUSSION

As revealed by this study, the endothelium of CAM blood vessels undergoes a sequence of structural changes as the membrane ages. Most prominently the cell nucleus, which at 8 days is extremely flat with little chromatin, becomes progressively rounder, increases its content of condensed chromatin and develops, by 18 days of incubation, an irregular contour with many invaginations of the nuclear membrane. Simultaneously, the frequency of various cell organelles changes as exemplified by the appearance of Weibel-Palade bodies, an increased abundance of pinocytotic vesicles and a decrease in the amount of rough endoplasmic reticulum and free ribosomes. With regard to their morphology, the endothelial cells of the CAM up to day 10 of incubation resemble those of developing allantoic blood vessels (Sethi and Brookes, 1971) from which the CAM vessels are derived.

The endothelium of the CAM prior to 12 days of incubation also exhibits many of the characteristics ascribed to immature, recently formed endothelial cells in other tissues. Cliff (1963) in a study of new vessels in healing tissue in the rabbit ear

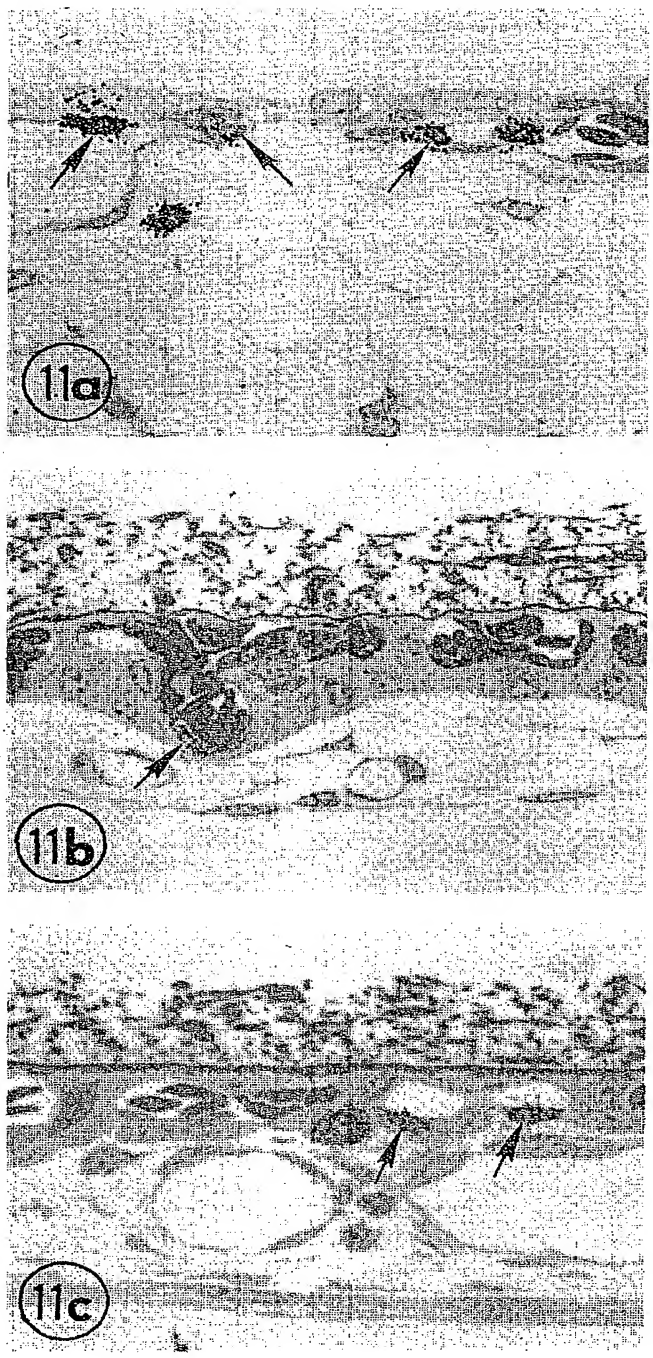

FIG. 11. Autoradiographs of the capillary network of the CAM after exposure to tritiated thymidine. (a) In the 10-day CAM, several endothelial cells are labeled (arrows). Other labeled cells include a chorionic ectodermal cell, a connective tissue cell and a blood cell. (b) At 11 days incubation, labeled endothelial cells (arrow) are rarely found. Other cells of the membrane are also infrequently labeled. (c) In the 14-day CAM, two labeled endothelial cells (arrows) are situated near a labeled ectodermal cell. × 690.

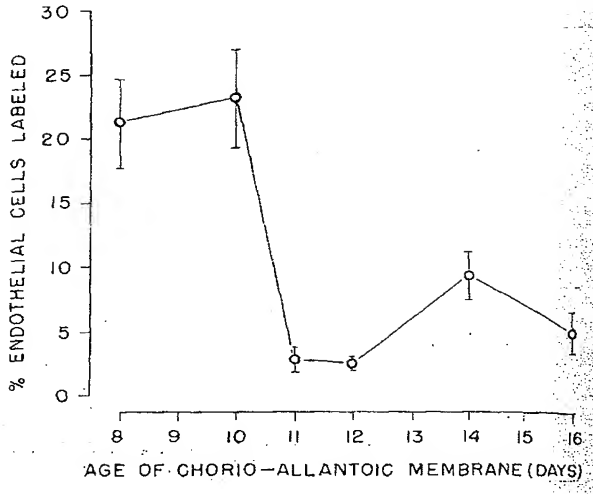

FIG. 12. Thymidine labeling index of endothelial cells in the chick CAM as a function of age of the embryo. Bars represent standard deviations.

chamber describes endothelial cells as possessing plentiful rough endoplasmic reticulum and a prominent Golgi apparatus. Schoefl (1963) has demonstrated that the endothelium of growing capillaries also contains many free ribosomes, few pinocytotic vesicles and exhibits a discontinuous or absent basement membrane. By contrast, mature endothelial cells contain numerous pinocytotic vesicles, little rough endoplasmic reticulum, few free ribosomes (Majno, 1965) and frequently, Weibel Palade bodies (Weibel and Palade, 1964). These characteristics of mature endothelium are acquired by CAM endothelial cells only after 12 days of incubation. When comparing the ultrastructure of chick CAM blood vessels and their precursors in the allantois, Sethi and Brookes (1971) concluded that whereas allantoic endothelium appears immature, that of CAM vessels has differentiated into mature endothelial cells. In contrast to the observations in the present study, however, maturity was reportedly reached by 6 days of incubation and further changes were not seen as the membrane aged.

From the variation in the percentage of endothelial cells incorporating tritiated thymidine as the CAM ages, it appears

that prior to 11 days of incubation blood vessels are rapidly growing as a result of continual endothelial cell mitosis. After 11 days, the volume of the CAM capillary network appears to increase very little as evidenced by the sharp decrease in the percentage of labeled endothelial cells. The rapid growth of the network of CAM vessels up to 11 days of incubation probably reflects the continued expansion of the chorioallantois so as to eventually encompass the contents of the fertilized egg and surround the albumen (Hamilton, 1952). By 12 or 13 days the chorioallantois lines the entire shell membrane and its expansion is complete (Romanoff, 1960).

Since labeled endothelial cells were distributed evenly throughout the capillary bed, this network prior to 11 days of incubation appears to be expanding by an overall proliferation of the endothelial cells in existing capillaries. This method of blood vessel growth can be contrasted with the initial formation of vessels in the allantois on day 4 of incubation by a lateral linkage of neighboring mesodermal cells (Sethi and Brookes, 1971). Although the advancing edge of the CAM was not studied, it appears that the extension of CAM vessels in the expanding membrane prior to 11 days is accomplished by lengthening of the existing vascular network rather than by formation of new capillary sprouts as occurs in wound healing (Clark and Clark, 1939; Cliff, 1963) or vascularization of tumor transplants (Algire and Chalkley, 1945; Warren and Shubik, 1966). A similar method of vessel growth by endothelial cell mitosis, resulting in lengthening of preexisting vessels, reportedly occurs in healing wounds prior to sprout formation (Chalkley *et al.*, 1946; Schoefl, 1963) and in iris vessels located several millimeters from intraocular tumors (Gimbrone *et al.*, 1973).

The high labeling index of endothelial cells in the CAM and their lack of maturity prior to day 11 may help to explain the relationship between the age of the CAM and its ability to support grafted tissue. The membrane is highly vascular at all stages in its development, and the lack of growth of tissue transplanted to the young chorioallantois has generally been attributed to the distance of the capillary bed from the membrane surface (Danchakoff, 1918; Romanoff, 1960). It seems possible that the growth of transplants may also depend upon the state of development of the host tissues onto which the transplants are grafted.

In summary, it appears that prior to day 11, the endothelium of the chick CAM is a rapidly dividing population of relatively undifferentiated cells, whereas after day 11, endothelial cells divide only infrequently and gradually acquire the morphological characteristics of differentiated endothelial cells.

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